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Laser-Induced Fluorescence Studies of Normal and Malignant Tumour Tissue of Rat Following Intravenous Injection of δ-Amino Levulinic Acid

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Background and Objective: **Laser-induced fluorescence was studied in normal and tumour tissue of rat after intravenous injection of δ-amino levulinic acid (ALA). The aim of the study was to investigate the protoporphyrin IX accumulation in different tissue types in rat after systemically administered ALA.**
Study Design/Material and Methods: **A malignant rat tumour and normal tissue from 13 different organs were investigated in eight rats. The rats were injected with two different ALA doses, 30 and 90 mg/kg b.w., and the investigations were performed at 10, 30, and 240 min after the injection. The fluorescence was recorded utilising an optical fibre based fluorosensor at 405 nm excitation.**
Results: **Fluorescence spectra were recorded in the 400–750 nm wavelength region including the dual-peaked PpIX fluorescence at about 635 and 705 nm, and the tissue autofluorescence peaking at about 500 nm. The maximum tumour build-up of PpIX was achieved already in less than 1 hr after ALA injection. The fluorescence demarcation between tumour and surrounding tissue was a factor of 7–8:1 after 30 min and decreased for longer retention times. The accumulation in 13 different organs was investigated and a particularly high PpIX build-up was found in stomach and intestine.**
Conclusions: **Fluorescence detection following i.v. injection of ALA provides attractive diagnostics for the experimental tumour used, indicating clinical usefulness. Lasers Surg. Medicine 20: 272–279, 1997.** **© 1997 Wiley-Liss, Inc.**

Key words: δ-aminolevulinic acid; laser-induced fluorescence; photodynamic therapy; protoporphyrin IX

INTRODUCTION

Optical techniques have become increasingly useful for medical diagnostics. Laser-induced fluorescence (LIF) [1], Raman scattering [2], and optical transillumination [3,4] have found many applications and are now used in clinical research. The most common of the optical techniques in medicine is laser-induced fluorescence, which is frequently used for detection of malignant tumours [5,6]. LIF has the advantages of a high sensitivity, is non-destructive, can be performed with the aid of optical fibres [7] and can be adapted for real-time imaging of tissue [8,9]. Most often, fluorescence detection of tumours is performed with the aid of an exogenous fluorophore

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that is, to a certain degree, localised in malignant tumours after administration to the patient.

The most important substance has been hematoporphyrin and its derivatives, in particular Photofrin. These agents have been extensively used for photodynamic therapy (PDT) of a variety of malignant tumours [10–13]. Haematoporphyrin gives rise to a dual-peaked fluorescence structure in the red region of the spectrum and is therefore easy to distinguish from other fluorescence structures. Furthermore, it is to some extent selectively localised in tumour tissue, although many doubts have been raised on this matter [14].

Substantial research work has been invested in finding new tumour localisers with more optimal characteristics for PDT and fluorescence detection. In particular, the low selectivity and long retention in the skin are severe drawbacks of Photofrin. Phthalocyanines [15], chlorins [16], benzoporphyrins [17], and carotenoporphyrins [18] are a few of a large number of substances tested with a varying degree of success in PDT and fluorescence detection. One of the most promising new agent is δ-amino levulinic acid (ALA). ALA is a natural precursor to protoporphyrin IX (PpIX) in the haem biosynthesis pathway of biological systems [19]. In normal cells, the synthesis of haem and the precursor PpIX is regulated by a feedback control system. It has been suggested, that for malignant cells this feedback system can be set out of control if a high amount of ALA is applied to the system. This has been successfully utilised for PDT [20,21] and for fluorescence detection [21–25] of tumours. For skin tumours, ALA is normally applied topically, and for application in the urinary bladder, instillation has been used. Recently, also oral administration has proven to be very useful, *e.g.,* for use in the larynx [24].

This article reports on intravenous (i.v.) injection of ALA with application to fluorescence detection of malignant tumours. Fluorescence spectra from rat tumours and their surroundings were recorded for different ALA doses and application times. The fluorescence from some inner organs was also investigated in order to study the clearance of ALA-induced PpIX. A set-up for clinical recording of fluorescence was used to record fluorescence spectra from 400 to 750 nm. Using UV or blue light excitation, the fluorescence from endogenous chromophores, the autofluorescence, could be evaluated. As the autofluorescence in many cases differs in tumour and its surroundings, this can enhance the tumour demarcation if incorporated in the fluorescence analysis. Thus, it has been found that the autofluorescence in the region of 400–500 nm is generally lower for malignant tissue than for normal surrounding tissue [26]. A lowered autofluorescence was reported for tumours, e.g., in the skin, lung parenchyma, brain cortex and white matter, and mouth mucosa [7,21]. Furthermore, by using a ratio of fluorescence intensities, dimensionless quantities are obtained, which are insensitive to target distance and excitation light intensity fluctuations, which is an extremely important aspect for clinical use.

MATERIALS AND METHODS

The ALA (Porphyrin Products Inc., Logan, UT, Lot 020592) was dissolved in sterile water to a concentration of 4 mg/ml and injected intravenously in *vena Femoralis.* Six Wistar/Furth rats received 30 mg/kg b.w. and two received 90 mg/kg b.w. of ALA. The animals had one tumour subcutaneously inoculated in both hind legs 9 days prior to the investigation. The tumour cell line was a colon adenocarcinoma (DMH-W49) chemically induced by injection of dimethylhydrazine [27]. One animal was sacrificed 10 min (30 mg/kg b.w.), four animals were sacrificed 30 min (3: 30 mg/kg b.w.; 1: .90 mg/kg b.w.), and three animals were sacrificed 4 hrs. (2: 30 mg/kg b.w.; 1: 90 mg/kg b.w.) after the ALA injection.

At the time of the fluorescence measurements, the tumours were 8–10 mm in diameter. The fluorescence investigation was performed directly after the sacrifice of the animals. The hind legs of the rats were carefully shaved to avoid the strong fluorescence from hair and the skin was removed from the tumour and the surrounding muscle area. The fluorescence was recorded in scans of about 10 points across the tumour area including the tumour capsule and healthy muscle on both sides of the tumour. The tumours were also cut in two halves and vertical scans from the top of the capsule to healthy muscle underneath the tumour were recorded. In addition, the abdomen was cut open and the fluorescence from liver, spleen, kidney, stomach, large and small intestine, abdominal wall, heart, and lung was measured. Fluorescence spectra were also taken from the trachea and the urinary bladder.

The fluorescence investigation was performed with a fibre-guided optical multichannel analyser (OMA) system. The excitation source was a nitrogen laser-pumped dye laser (Laser Science, VSL-337 and VSL-DCM-3, respectively)

tuned to 405 nm. The pulse length was 3 ns and the output pulse energy of the dye laser was about 20 μJ. The excitation light was focused via a dichroic mirror onto the tip of a 600 μm diameter low-fluorescence quartz fibre (Fiberguide Ind., SFS600N). As the fibre tip was held in contact with the tissue, the induced fluorescence was guided back through the same fibre and focused onto the entrance slit of a spectrometer (Jarrel Ash, Monospec 27). A 1,024 channel image-intensified diode array detector (EG&G PARC OMA III) was utilised to record the fluorescence spectrum ranging from 400 to 750 nm. A cut-off filter (Schott, GG435) was placed in front on the entrance slit to prevent any scattered laser light from reaching the detector. The fluorescence from 100 laser shots was integrated for each spectrum to obtain a high signal-to-noise ratio. The spectra were displayed on a screen and stored for evaluation. A cuvette with 3 mg/ml Rhodamin 6G in ethylene glycol was used as an intensity standard and used before and after investigation of each animal. The fluorosensor system was previously described in Ref. 7.

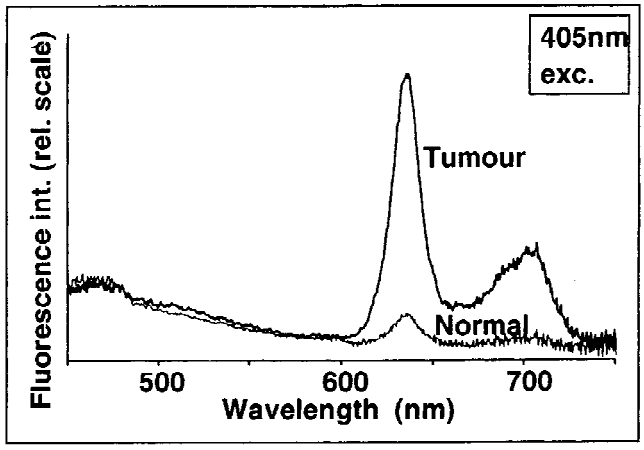

Fig. 1. Typical fluorescence spectra from a Wistar/Furth rat injected i.v. with 30 mg/kg b.w. of ALA dissolved in distilled water. The tumours are colon adenocarcinoma grown at the hind leg of the rat. The two spectra were measured on the tumour capsule and 10 mm from the tumour, respectively. The excitation wavelength was 405 nm.

RESULTS

Figure 1 shows examples of fluorescence spectra from tumour exterior and normal muscle tissue recorded after removing the skin. The two fluorescence features peaking at 635 and 705 nm originate from the ALA-induced PpIX. In addition, a broadband autofluorescence peaking at about 500 nm was observed. As can be seen, the PpIX fluorescence is much more pronounced in the tumour compared with the surrounding muscle tissue. The autofluorescence, on the other hand, is in this case comparable for the two spectra and gives no contribution to the tumour demarcation for fluorescence diagnostics.

Fluorescence spectra were recorded 30 min post-ALA injection for the tumour region and some inner organs, and the fluorescence intensity was evaluated at the PpIX peak at 635 nm as well as at 500 nm corresponding to the autofluorescence of the respective tissues. The fluorescence intensity at 635 nm for the different organs, normalised to the fluorescence intensity for the tumour exterior, are presented in Figure 2. A dashed line at a ratio equal to 1 representing tumour exterior is inserted as a guidance for the eye. The data represents a mean value of three measured animals with the corresponding standard deviation given for each value. As can be seen, the gastrointestinal organs exhibit very pronounced fluorescence with values of up to 10 times more than that from tumour tissue. The excretion organs, liver, spleen, and kidney, on the other hand, show very weak fluorescence at 635 nm.

Figure 3 shows a fluorescence scan across a tumour region. The scan starts on normal muscle tissue 10 mm from the tumour border, continues over the tumour capsule, and ends on normal muscle 10 mm from the tumour. The fluorescence spectra shown in Figure 3 are all in the same intensity scale and thus comparable in that respect. The scan shows a very homogenous distribution of PpIX in the tumour while the amount of autofluorescence varies across the scan. This variation is probably related to the pattern of collagen-rich connective tissue. In addition, a satellite peak appears in some of the spectra at 590 nm. This additional peak is not present in the control animals, which had not received any ALA.

After the tumour exterior had been investigated, the tumours and their surroundings were cut open and fluorescence spectra were recorded at different depths in the tumour region. Figure 4 shows such a scan starting at the tumour capsule, going down through the tumour, and a few mm into normal muscle tissue beneath the tumour. Also in these scans, a high PpIX production is measured in the tumours as an increased fluorescence at 635 nm. In addition, the highest PpIX fluorescence was found close to the border region in malignant tissue. The recorded autofluorescence in the 500 nm spectral region was lower in

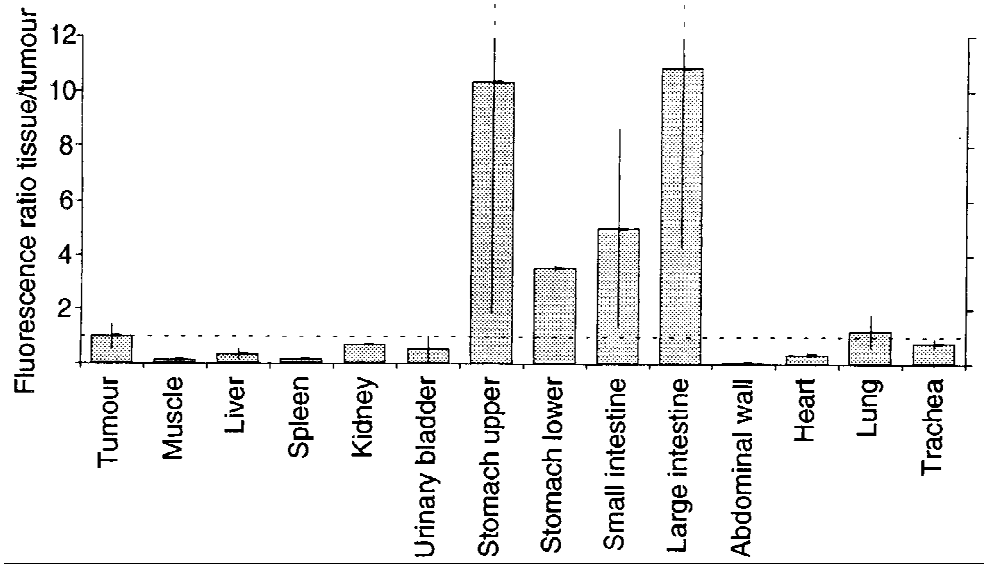

Fig. 2. Fluorescence from tumour, muscle, liver, spleen, kidney, urinary bladder inside, stomach outside, small and large intestine outside, abdominal wall, heart, lung outside, and trachea inside of Wistar/Furth rat. The fluorescence is plotted as a ratio normalised to the fluorescence from tumour exterior. The data was evaluated at 635 nm. A dashed line at ratio 1 representing tumour is inserted as a guidance for the eye. The ALA dose was 30 mg/kg b.w. and all six rats included in this figure were investigated 30 min post-injection.

the vertical scans as compared to the superficially recorded spectra.

Figure 5 shows the fluorescence intensity at 635 nm for tumour exterior and muscle tissue for three different drug retention times. The intensity is expressed in units relative to the Rhodamin fluorescence standard. The plotted values are mean values based on data from six rats injected with 30 mg/kg b.w. of ALA. For each measured rat, about 20 fluorescence spectra were recorded. The error bars indicate one standard deviation. The maximum retention is achieved already 1 hr or less post-injection. In addition to the above mentioned measurements with 30 mg/kg b.w., two rats were injected with 90 mg/kg b.w. However, the tumour-to-normal ratio was not higher, and the lower dose was therefore judged to be the preferred. The ratios of the 635 nm fluorescence for tumour exterior/muscle and tumour interior/muscle are presented in Figure 6. The tumour demarcation is at its maximum almost directly after injection and has decreased to half its maximum after about 4 hrs, although the data spread is quite large.

DISCUSSION

ALA for clinical use has increased drastically during the past few years due to a comparatively high tumour selectivity and a rapid clearance from the human body. Topical application of ALA has led to a high success rate for PDT and fluorescence diagnostics. One potential disadvantage of topical application is a poor penetration into certain tumour types. Thus, the PDT response in recurrent breast carcinoma after topically applied ALA has been reported to be very poor [28,29]. Here we show that systemic administration of ALA is a good alternative to topical application, resulting in a high tumour contrast at early times after injection.

The distribution pattern for i.v. ALA, as shown in Figure 2, indicates a high PpIX level in the gastrointestinal organs, up to 10 times more than in tumours. The liver, on the other hand, showed a surprisingly low level of porphyrins, less than half of that in tumours. In the literature, very high values of liver/tumour ratios have been reported (10 or more) [30,31]. However, available data are acquired 1 hr or more post-injection and very few investigations have reported results for shorter retention times. The investigation also included one animal with 4 hrs retention time (data not shown) and in that case the liver/tumour ratio was about 7. This is in agreement with earlier reports [30,31]. Furthermore, for one animal investigated 10 min post-injection, a very low porphyrin fluorescence was

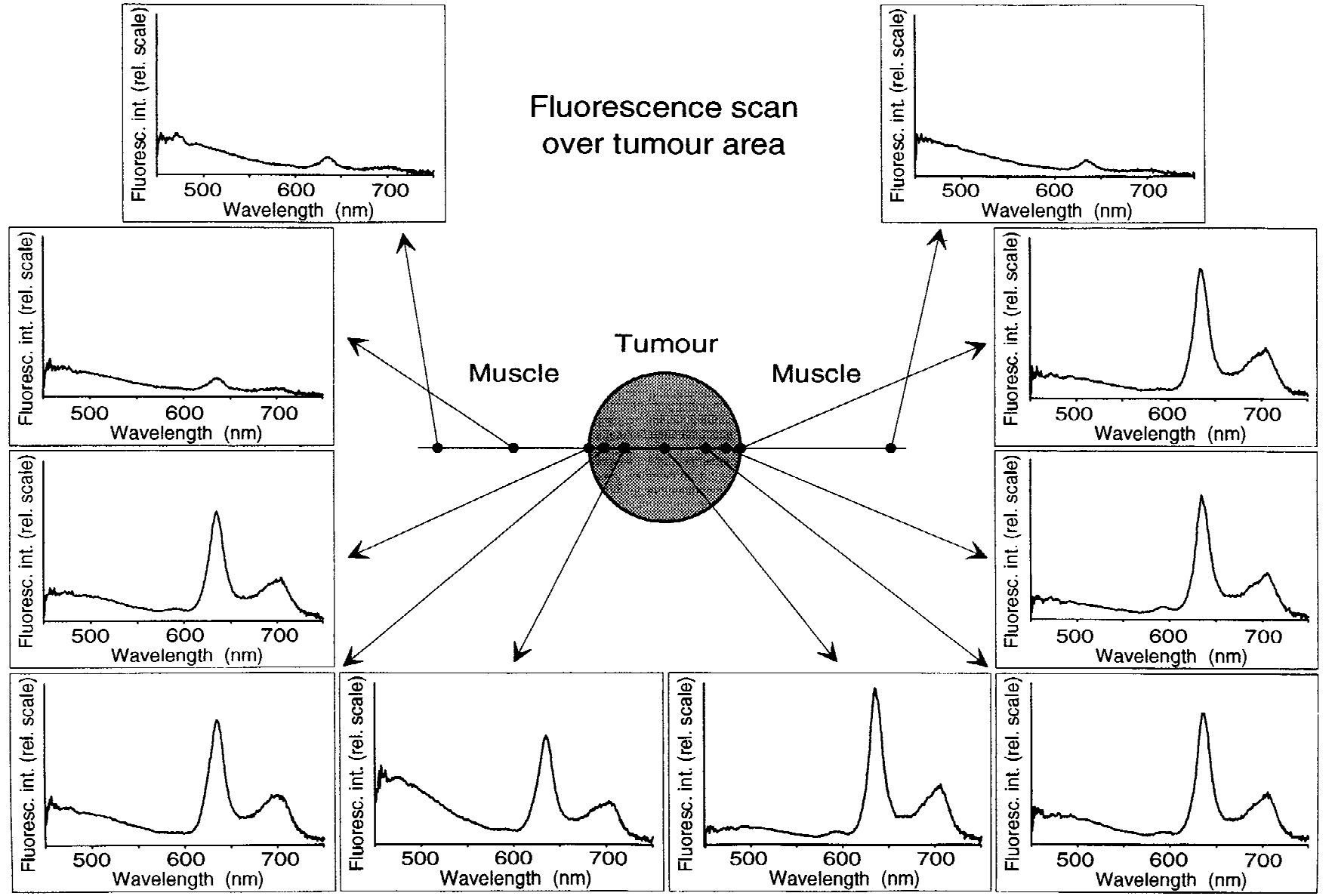

Fig. 3. Fluorescence scan across a tumor area with the skin removed. The scan starts in normal muscle tissue 10 mm from the tumour, goes onto the tumour capsule, and onto normal muscle tissue again. The corresponding fluorescence spectra are shown for each measured point.

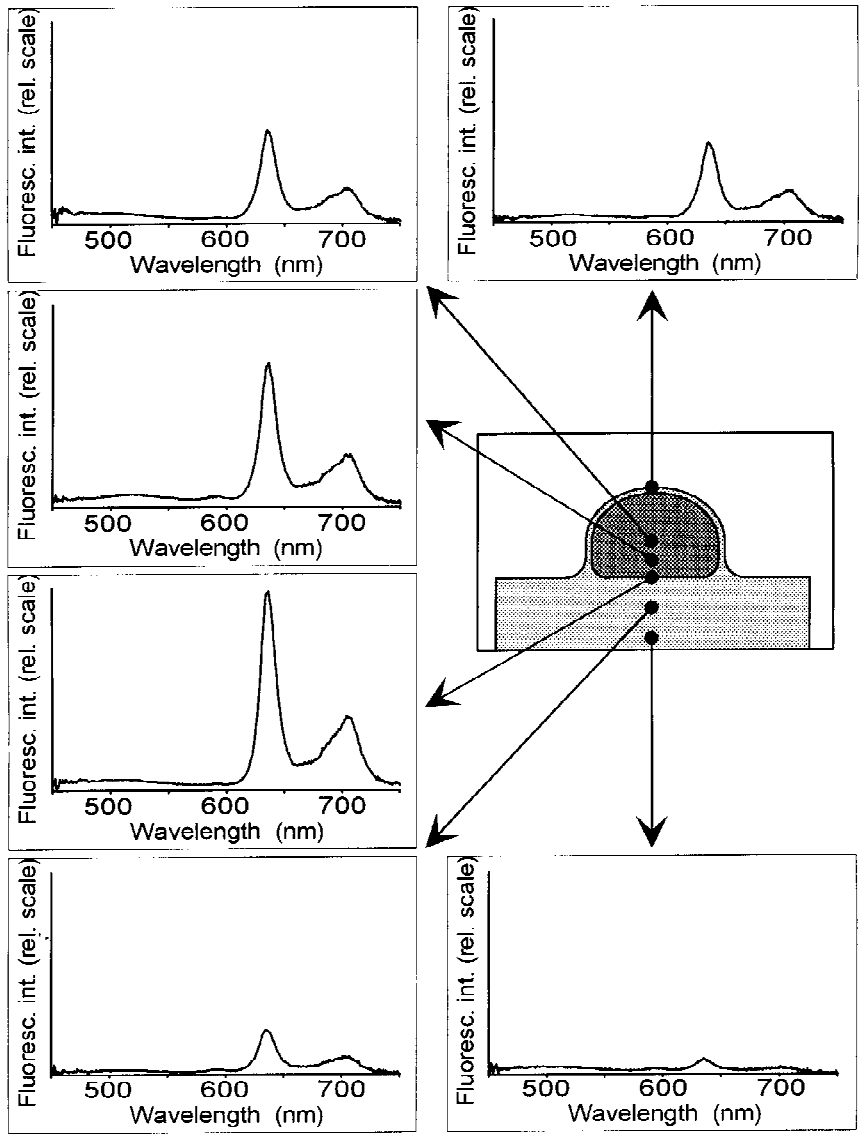

Fig. 4. Vertical fluorescence scan into a tumour region. The scan starts at the top of the tumour capsule, goes into the tumour, and further into normal muscle tissue underneath the tumour.

found in liver, indicating a slow PpIX synthesis in the liver.

The uptake and retention in this Wistar/Furth colon tumour monitored by LIF has been reported for several other photosensitises in an earlier study [32]. In general, the distribution pattern for ALA induced porphyrins is similar to that of haematoporphyrin i.v. injected animals with 2 hrs retention time. This is not surprising, as ALA as well as hematoporphyrin are relatively hydrophilic. The important exception for ALA is the much higher amount of porphyrins in tumours than in the surrounding muscle tissue. The fact that the kinetics of PpIX synthesis is different for tumours and liver suggests that the porphyrins detected in tumours 30 min post-injection is mainly synthesised in the tumours, rather than in the liver and then transported to the tumours. This is in good agreement with other reports [30]. Another argument in this direction is the fact that a substantial amount of synthesised PpIX is found already in tumours (Fig. 5) 10 min post-injection.

The highest amount of detected PpIX in tumours was found 30 min post-injection, while for muscle tissue, no clear peak could be observed.

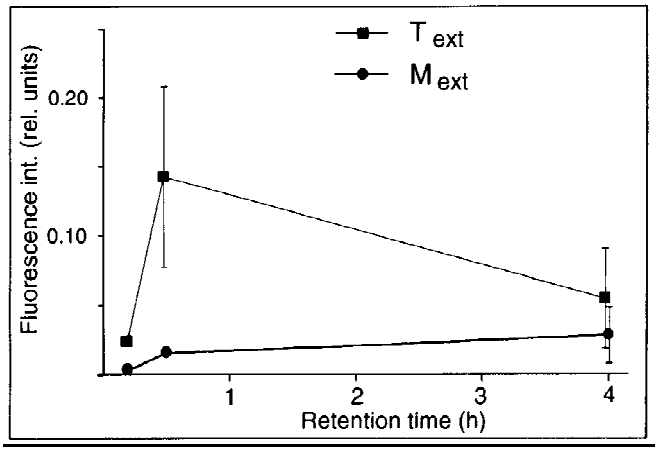

Fig. 5. Fluorescence intensity at 635 nm as a function of time between ALA injection and measurement. For each data point, the mean value from approximately eight spectra and ±1 standard deviation are given. The data for tumours and normal muscle are indicated with squares and circles, respectively. The ALA dose was 30 mg/kg b.w. for all animals.

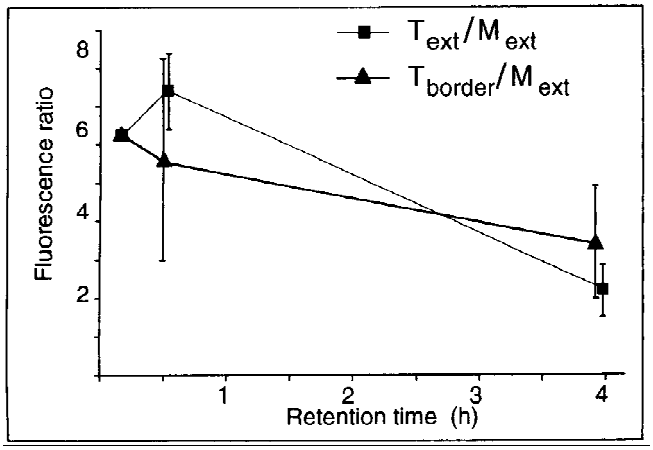

Fig. 6. Tumour to normal fluorescence ratio at 635 nm as a function of time between ALA injection and measurement. For each data point, the mean value from approximately eight spectra and ±1 standard deviation are given. Data are given for tumour exterior to normal (squares) and for tumour exterior border to normal (triangles). The ALA dose was 30 mg/kg b.w. for all animals.

The maximal tumour exterior to muscle ratio was also found at 30 min post-injection with a ratio of 7–8. This is in good agreement with other reports on i.v. injected ALA. The fact that a high tumour/normal ratio can be obtained already after less than half an hour may have important implications for fluorescence diagnostics of tumours. Taking also into account that the ALA induced PpIX is more or less cleared from the system in about 24 hours, i.v. injected ALA seems to be a very useful alternative for fluorescence diagnostics or PDT. For most tumour types, additional tumour contrast can be gained by including the tissue autofluorescence into the diagnostic criterion.

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